Extremophiles (2005) 9: 407–414
DOI 10.1007/s00792-005-0454-3

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# Microbial community in a geothermal aquifer associated with the subsurface of the Great Artesian Basin, Australia

Received: 7 January 2005 / Accepted: 8 April 2005 / Published online: 25 June 2005


**Abstract** To investigate the biomass and phylogenetic diversity of the microbial community inhabiting the deep aquifer of the Great Artesian Basin (GAB), geothermal groundwater gushing out from the aquifer was sampled and analyzed. Microbial cells in the groundwater were stained with acridine orange and directly counted by epifluorescence microscopy. Microbial cells were present at a density of $10^8$–$10^9$ cells per liter of groundwater. Archaeal and bacterial small-subunit rRNA genes (rDNAs) were amplified by PCR with *Archaea*- and *Bacteria*-specific primer sets, and clone libraries were constructed separately. A total of 59 clones were analyzed in archaeal and bacterial 16S rDNA libraries, respectively. The archaeal 16S rDNA clones were divided into nine operated taxonomic units (OTUs) by restriction fragment length polymorphism. These OTUs were closely related to the methanogenicgenera *Methanospirillum* and *Methanosaeta*, the heterotrophic genus *Thermoplasma*, or miscellaneous crenarchaeota group. More than one-half of the archaeal clones (59% of total 59 clones) were placed beside phylogenetic clusters of methanogens. The majority of the methanogen-related clones (83%) was closely related to a group of hydrogenotrophic methanogens (genus *Methanospirillum*). The bacterial OTUs branched into seven phylogenetic clusters related to hydrogen-oxidizing thermophiles in the genera *Hydrogenobacter* and *Hydrogenophilus*, a sulfate-reducing thermophile in the genus *Thermodesulfovibrio*, chemoheterotropic bacteria in the genera *Thermus* and *Aquaspirillum*, or the candidate division OP10. Clones closely related to the thermophilic hydrogen-oxidizers in the genera *Hydrogenobacter* and *Hydrogenophilus* were dominant in the bacterial clone library (37% of a total of 59 clones). The dominancy of hydrogen-users strongly suggested that $H_2$ plays an important role as a primary substrate in the microbial ecosystem of this deep geothermal aquifer.

**Keywords** Great Artesian Basin · Deep aquifer · Geothermal groundwater · Hydrogen-oxidizer · Methanogen · Subsurface biosphere

Communicated by K. Horikoshi

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The Great Artesian Basin (GAB) is the largest artesian groundwater basin in the world occupying over 1.7 million $km^2$, and it covers more than one-fifth of the Australian continent (reviewed by Habermehl 1980). The GAB consists of alternating layers of water-bearing permeable sandstone and no water-bearing impermeable shale. These layers of GAB were formed between 100 and 250 million years ago (Senior et al. 1978). Groundwater in the aquifer is mainly recharged by rainfall infiltrating into outcrops that occur primarily along the western slope of the Great Dividing Range (GDR) in the north-east margin of the GAB. The water flows down through the sandstone outcrops, and then the water is naturally reserved in the deep aquifer of the GAB. The deep aquifer is warmed by non-volcanic geothermal heat. The geothermal gradients range from

15.4°C/km to 102.6°C/km, with a mean of 48°C/km (Polak and Horsfall 1979). The deep aquifer shows extremely high water pressure and high temperature; therefore, a vast amount of geothermal groundwater gushes out whenever a well is drilled to depth of the deep aquifer. The accumulated discharge rate of these artesian wells in the GAB is about $1.5\times10^6$ $m^3$ $day^{-1}$ (Seidel 1980).

The geothermal groundwater flowing from artesian wells is temporarily stocked in bore water pools and is made to flow in artificial channels. The water in the channels is used for the maintenance of a major pastoral industry. Prolific microbial mats were frequently seen in the hot water of the bore pool and channels. A large number of thermophiles have been identified and isolated from the mats, for example, thermophilic cyanobacteria, hydrogen-oxidizers, *Thermus* species, *Thermotogales* species, clostridia, and species in the beta- and delta-Proteobacteria (e.g., Byers et al. 1998; Spanevello et al. 2002). While it has vigorously studied on the microbial community in the bore water pool and the artificial channels on the ground surface where exposed to the air and sunlight, no ecological studies of the microbial communities in the outflow, or the geothermal aquifer of the GAB have been reported. Little is known about both the biomass and phylogenetic diversity of the subsurface microbial ecosystem.

Some microbiological studies associated with the subsurface environments of geothermal or hydrothermal areas such as hot springs and hydrothermal vents have been conducted using samples of sediment, rock core, geothermal groundwater, and hydrothermal fluid obtained from such environments (e.g., Kimura et al. 2003; Marteinsson et al. 2001; Summit and Baross 2001; Takai et al. 2004). However, hot springs and hydrothermal vents are generally unstable, and the thermal conditions fit for living microorganisms rarely last long enough, due to violent fluctuations in the energy supply. In contrast to these unstable environments, the geothermal GAB-aquifer is far more stable. The groundwater in the GAB flows at a rate ranging between 1 and 5 m per year, and the age of the oldest groundwater in the aquifer is estimated to be 2 million years (Elmore et al. 1979; Collon et al. 2000). The extremely slow rate of flow and geothermal heating render the GAB a stable environment suitable for thermophilic microbes to prosper on a geological timescale. Here, we present the first evidence of the occurrence of a microbial community in the geothermally stable environment of the GAB, with a discussion of its biomass and phylogenetic diversity.

Groundwater samples were collected from an artesian well (registered bore number 378; 24°37′16S, 145°19′46E) situated near Blackall in western Queensland, Australia. This artesian well has a depth of 936.7 m, and steel casing pipes are inserted to near the bottom (a depth of 935.7 m) inside the bore hole to prevent the inflow of groundwater from shallow layers. The flow rate of the water at the bore source is approximately 11 l/s (reported by the Department of Natural Resources, Mines and Energy of the Queensland government). The extremely rapid water flow is likely not to permit formation of microbial mats inside the casing pipes. Therefore, the bore water probably reflects the environment and microbial community within the deep geothermal aquifer, not in the drilled bore holes. The water left the bore at a temperature of 64.4°C, pH of 8.0, and ORP of −48 mV. These parameters remained unchanged from those measured at least 40 years ago.

The groundwater samples were collected from the bore by using sterile silicon tubes and bottles for direct cell count and DNA analysis. The silicon tubes were placed deeply into the artesian well via a hose to prevent sample contamination. The bottles were pre-washed and pre-rinsed with sterilized distilled water, and then were re-washed with the groundwater before sample collection. A 1.0 ml sample of groundwater was filtered using pre-blackened polycarbonate filters (pore size, 0.22 μm; diameter, 25 mm; Millipore Corp.). Microbial cells were collected on the filter and stained with 0.01% acridine orange in 10 mM phosphate buffer (pH 8.0) for 5 min (Hobbie et al. 1977). The microbial cells were observed by epifluorescence microscopy and counted over 40 microscopic fields. The microbial cell density in the geothermal groundwater was $10^8$–$10^9$ cells per liter.

Exactly 6 l of the groundwater was aseptically filtrated with a housing filter (Sterivex-GV; pore size, 0.22 μm; Millipore Corp.) via a sterile silicon tube and a peristaltic pump, and bulk DNA of microbes collected on the filter was extracted by a method described by Somerville et al. (1989). A concentration of the bulk DNA was measured by using a UV-spectrometer (Beckman Coulter, Inc.); $1.5\pm0.4$ μg DNA per liter of groundwater was estimated from the resultant DNA concentration and a volume of the filtered groundwater. The total cell density inferred from the DNA concentration (2.5 fg of genomic DNA per microbial cell; Button and Robertson 2001) was $6.0\pm1.6\times10^8$ cells per liter of groundwater. This estimation roughly corresponded to the total cell density obtained by direct cell count. These results suggest that the microbial cell density in the geothermal groundwater was almost equal to that observed in coastal seawater (Kimura et al. 1999).

The archaeal and bacterial 16S rDNAs in the bulk DNA were quantified by the quantitative fluorescent PCR method as previously described (Takai and Horikoshi 2000). An archaeal rDNA amplified from *Haloferax denitrificans* (almost the full length between primer Arch21F (DeLong 1992) and Uni1492R (Lane 1991)) was used as standard rDNA. The PCR analysis and the fluorescence signal monitoring were performed using an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems), and the threshold cycles of the PCR amplification were analyzed with 7300 system SDS software (Applied Biosystems). The contents of the archaeal and bacterial 16S rDNAs in the geothermal groundwater were shown in Table 1. The percentage of

**Table 1** Quantification of archaeal and bacterial rDNA in bulk DNA obtained from a microbial community in geothermal groundwater

| | |
|---|---|
| Temperature | 64.4°C |
| pH | 8.00 |
| Amount used for DNA extraction | 6 l |
| Cell density[a] | $10^8$–$10^9$ cells/l |
| DNA yield[b] | 1.5 ± 0.03 μg/l |
| Concentration of prokaryotic rDNA | 7.0 ± 1.4 pg/l |
| Concentration of archaeal rDNA | 1.3 ± 0.2 pg/l |
| Percentage of archaeal rDNA[c] | 18.1 ± 4.3% |
| Concentration of bacterial rDNA[d] | 5.7 ± 0.8 pg/l |
| Percentage of bacterial rDNA[e] | 81.9 ± 9.5% |

[a]Direct cell count as determined with acridine orange
[b]DNA concentrations were measured with a UV spectrophotometer
[c]Percentage of archaeal rDNA in whole prokaryotic universal rDNA
[d]The bacterial rDNA was calculated using value that was subtracted archaeal rDNA from prokaryotic universal rDNA
[e]Percentage of bacterial rDNA in whole prokaryotic universal rDNA

the archaeal rDNA corresponded to 18.1% of the amount of prokaryotic universal rDNA, which simultaneously indicated that the bacterial rDNA content was 81.9% of the prokaryotic universal rDNA. The quantitative PCR analysis showed that bacteria were numerically superior to archaea in the microbial community of the deep aquifer.

The archaeal and bacterial 16S rDNAs were PCR-amplified with the following two primer sets: Arch109F (Großkopf et al. 1998) and Arch915R (Stahl and Amann 1991) for the archaeal 16S rDNAs, and Bac27F (Lane 1991) and Uni1492R for the bacterial 16S rDNAs. Approximately, 0.8-kp and 1.4-kb of PCR fragments were amplified with these primer sets, respectively. The PCR products were cloned using the Zero BluntTOPO PCR cloning kit (Invitrogen Corp.), following the manufacturer's protocol, and clone libraries of archaeal and bacterial 16S rDNAs were constructed separately. In order to form operational taxonomic units (OTUs), the cloned inserts were grouped by restriction fragment length polymorphism (RFLP) using *Hae*III, which recognizes the 4-bp restriction sites (Promega Corp.). The resultant RFLP was analyzed by electrophoresis on 2% agarose gel. The PCR products showing the same restriction patterns by *Hae*III were analyzed again with other 4-bp recognizant restriction enzyme *Rsa*I (Promega Corp.), and the PCR products were then further divided into groups. Further, this process was repeated with another 4-bp recognizant restriction enzyme, *Msp*I (Promega Corp.).

Fifty-nine clones were analyzed in each archaeal and bacterial 16S rDNA library. These archaeal and bacterial 16S rDNA clones were divided into nine OTUs (GAB-A01 to GAB-A09) and seven OTUs (GAB-B01 to GAB-B07) based on the RFLP analysis. These OTUs were designated as follows: GAB-A01 to GAB-A09 (archaea) and GAB-B01 to GAB-B07 (bacteria), respectively (Figs. 1 and 2).

The sequences of representative 16S rDNA clones in each OTU were determined for both strands by using a capillary DNA sequencer, the CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc.). For sequencing reactions, the vector-specific primers (T7 and M13 reverse) and the following nested 16S rDNA-specific primers were used: 324R, 357F, 519R, 530F, 1114F, 1100R, and 1389R (Lane 1991); 803F (Stackebrandt and Charfreitag 1990).

The 16S rDNA sequences of the representative clones were checked for chimeric artifacts by the Chimera check program in the Ribosomal Database Project II (Maidak et al. 2001) and the clones were homology-searched in the DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/) using the programs FASTA (Lipman and Person 1985) and BLAST (Altschul et al. 1990). The sequence data were aligned with the CLUSTAL W package (Thompson et al. 1994), and used to construct a phylogenetic tree by the neighbor-joining algorithm (Saitou and Nei 1987) with TreeView (Page 1996). The 16S rDNA sequences determined in this study were registered in DDBJ as shown in Figs. 1 and 2.

The phylogenetic analysis revealed that the OTUs of the archaeal clone library belonged to the genera *Methanospirillum*, *Thermoplasma*, *Methanosaeta*, and miscellaneous crenarchaeota group (Fig. 1). GAB-A01 and A03 were phylogenetically included in a cluster of the genus *Methanospirillum*, which is known to be a methanogenic group able to utilize $H_2$ as an electron donor (Ferry et al. 1974). GAB-A06 and A08 belonged to a cluster of the genus *Methanosaeta*, which is known to be a methanogenic group able to utilize acetate as an electron donor (e.g., Kamagata et al. 1992; Patel 1992). Clone sequences belonging to the four methanogenic OTUs (GAB-A01, A03, A06, and A08) were equivalent to 59% of the total archaeal clone library (59 clones). In particular, the clones related to hydrogenotrophic methanogens (the genus *Methanospirillum*) accounted for 83% of the total methanogenic clones, and 49% of the total archaeal clones.

The OTUs GAB-A04 and A05 were closely related to the genus *Thermoplasma*, and accounted for 15% of the total archaeal clones. It has been reported that the genus *Thermoplasma* consists of heterotrophic or mixotrophic archaea that are able to use elemental sulfur as an electron acceptor (Darland et al. 1970; Segerer et al. 1988). Three OTUs (GAB-A02, A07, and A09) were found to belong to a cluster within uncultured crenarchaeotic groups, and the clone number of the OTUs was equal to 25% of the total number of archaeal clones. The heterotrophic and uncultured archaeal clones accounted for 41% of the enter library, and these clones were clearly lower in number than the methanogenic clones.

The phylogenetic analysis revealed that the OTUs of the bacterial clone library belonged to clusters of the genera *Hydrogenobacter*, *Aquaspirillum*, *Hydrogenophilus*, *Thermodesulfovibrio*, *Thermus*, and the uncultured candidate division OP10 (Fig. 2). GAB-B01 and B03 were closely related to the genus *Hydrogenobacter* in the *Aquificae* and the genus *Hydrogenophilus* in the

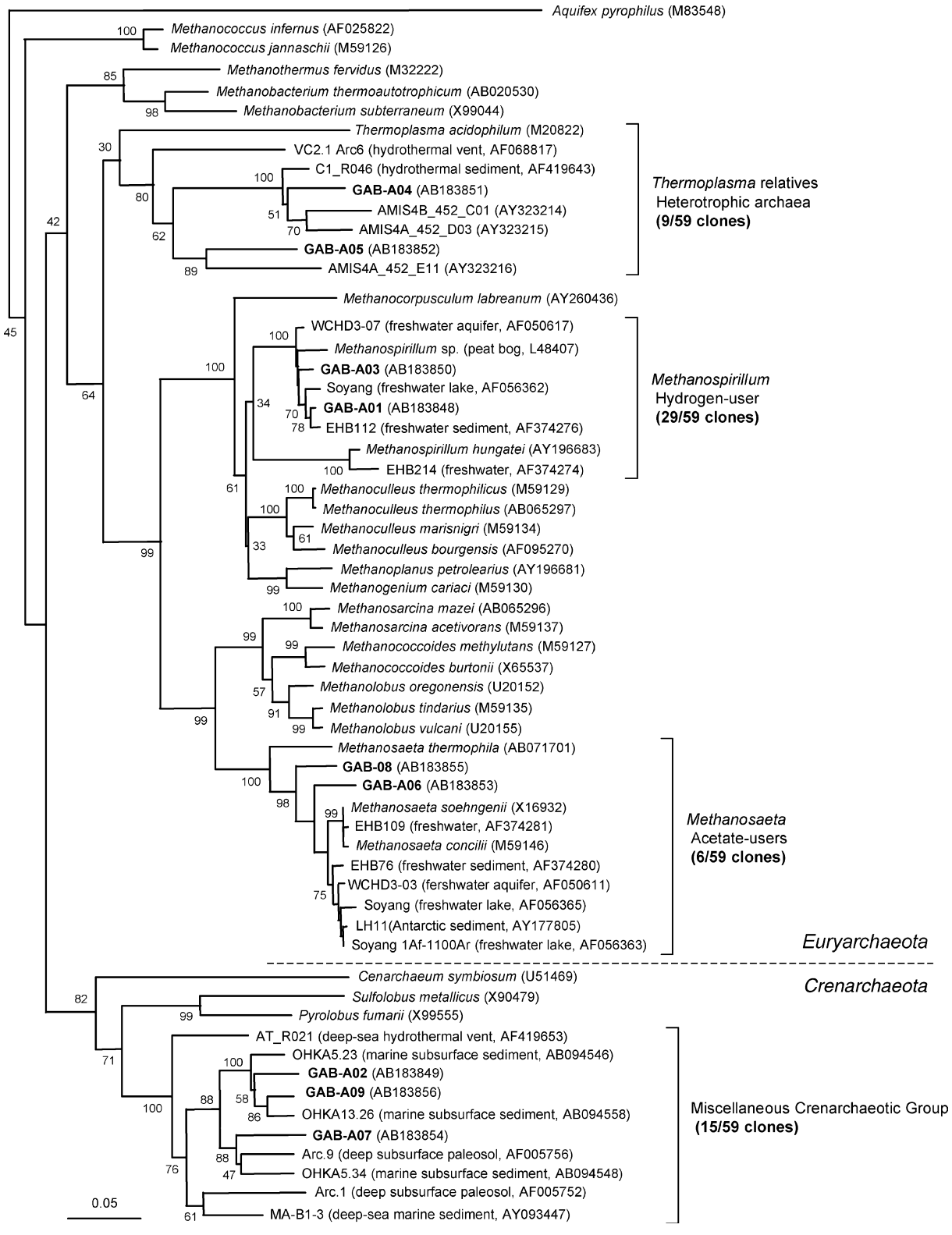


**Fig. 1** Molecular phylogenetic tree based on 16S rDNA sequences of nine representative clones in archaeal OTUs obtained from geothermal groundwater with the related species and environmental clones among the domain *Archaea*. The tree was constructed by a neighbor-joining analysis with ca. 710 bp homologous positions of the rDNA sequences. The values of 100 bootstrap trial replications are given next to the nodes of the trees. The scale bar represents 0.05 nucleotide substitutions per sequence position

beta-Proteobacteria, respectively. These genera are known to be primarily composed of hydrogen-oxidizing bacteria (HOB) isolated from geothermal environments such as hot springs and volcanic areas (Hayashi et al. 1999; Kawasumi et al. 1984; Shima and Suzuki 1993). The HOB-related OTUs (GAB-B01 and B03) were found to be dominant in the bacterial clone library, corresponding to 37% of the total clones in the library.

GAB-B04 and B05 belonged to a cluster of the genus *Thermodesulfovibrio* in the *Nitrospira*. The genus *Thermodesulfovibrio* consists of thermophilic sulfate-reducing bacteria (SRB), and most strains of this genus are isolated from thermal environments such as hot springs and thermal wastewater reactors (Henry et al. 1994; Sekiguchi et al. 1998). The SRB-related OTUs were accounted for 27% of the total bacterial clones.

GAB-B02 was closely related to the genus *Aquaspirillum* in the family Comamonadaceae (belonging to the beta-Proteobacteria). Since *Aquaspirillum* species and most members in the family Comamonadaceae are mesophilc, it is likely that GAB-B02 originated in a certain mesophile, possibly a contaminated bacterium during the sampling. However, it is known that some thermophilic species, e.g., *Thiomonas thermosulfata* and *Tepidimonas ignava* (Moreira et al. 2000), are also included in this family and interspersed among the mesophilic species. The interspersion of thermophiles within this family suggest that GAB-B02 was possibly obtained from a thermophilic Comamonadaceae species in the aquifer. The remaining OTU, GAB-B07, was clearly affiliated with the genus *Thermus*. All strains of this genus have been isolated from volcanic areas and hot springs (Chung et al. 2000; Hudson et al. 1987; Williams et al. 1996). All clones in these OTUs (GAB-B02 and B07) were considered to be obtained from obligatory heterotrophic bacteria as based on their phylogenetic relationship (the related genera *Aquaspirillum* (Wen et al. 1999) and *Thermus* contain no autotrophic species). Thus, all clones in these OTUs, taken together, accounted for 25% of the total clones.

Almost all of the OTUs identified in this study were closely related to the 16S rDNA sequences of thermophilic strains that had previously been collected from geothermal and hydrothermal areas. The following related species are known to grow at high temperatures: 45–63°C in the case of *Thermoplasma acidophilum* (Darland et al. 1970), 55°C–60°C (optimum temp.) in *Methanosaeta thermophila* (Kamagata et al. 1992), 37–80°C in *Hydrogenobacter thermophilus* (Kawasumi et al. 1984), 40–70°C in *Thermodesulfovibrio yellowstonii* (Henry et al. 1994), 50–80°C in *Thermus antranikianii* (Chung et al. 2000), and 50–52°C (optimum temp.) in *Hydrogenophilus thermoluteolus* (Hayashi et al. 1999).

Although culturable strains in the genus *Methanospirillum* are commonly mesophilic, many environmental clone sequences closely related to this genus were found in hot water samples (the number of this type was equal to a half of the total number of archaeal clones). Whether or not these clone sequences were retrieved from indigenous thermophilic methanogens remains open to discussion.

Several archaeal and bacterial OTUs were not related to any known species, but they were affiliated with environmental clone clusters such as miscellaneous crenarchaeotic group (GAB-A02, A07, A09) and bacterial candidate division OP10 (GAB-B06). Although there is no physiological information about two environmental clone clusters, these clusters mainly consisted of clones retrieved from hydrothermal, geothermal, or anaerobic environments (e.g., Chandler et al. 1998; Hugenholtz et al. 1998; Inagaki et al. 2003; Teske et al. 2002). This environmental information strongly suggests that these 'incertae sedis' clones were obtained from thermophilic microorganisms.

As mentioned above, all clones found in this study were considered to have originated from thermophiles that lived in subsurface environments of the GAB. Therefore, it appears quite probable that the results of the present analysis reliably reflect the microbial ecosystem in this geothermal aquifer.

In this study, epifluorescence microscopy showed an abundant biomass of microorganisms within a geothermal aquifer (the cell density was almost equal to that observed in coastal seawater). Additionally, the phylogenetic analyses indicated that OTUs belonging to hydrogenotrophic methanogens (genus *Methanospirillum*) and chemoautotrophic hydrogen-oxidizing bacteria (genera *Hydrogenobacter* and *Hydrogenophilus*) were dominant in archaeal and bacterial clone libraries, respectively. These chemoautotrophic microorganisms are known to use $H_2$ as an energy source, and they both generally play an important role as primary producers in various oligotrophic environments. The abundance of $H_2$-using microorganisms suggests that $H_2$ is the primary substrate in this deep subsurface ecosystem.

Assuming that this is true, a constant supply of $H_2$ would have to be necessary to maintain such an abundant microbial community. Apps and van de Kamp (1993) have mentioned some possible mechanisms for the generation of abiogenic $H_2$: (1) reactions between dissolved gases in magmas; (2) catalysis of silicates under stress in the presence of water; (3) radiolysis of water by radioactive isotopes of uranium, thorium, and potassium; (4) reactions between $CO_2$, $H_2O$, and $CH_4$ at elevated temperature in vapors; and (5) interaction of water with ferrous minerals in mafic rocks. In addition, $H_2$ can be also produced by thermocatalysis of organic materials when deep-sea hydrothermal fluids interact with organic-rich sediments (Welhan and Lupton 1987; Morita 2000). The geothermal GAB-aquifer is surrounded by layers of impermeable shale containing abundant organic materials (Habermehl 1980). It is quite possible that $H_2$ is produced by interactions between the geothermal groundwater and organic-rich shale. It is also possible that $H_2$ is generated by the upwelling from magmatic material in the deeper layers and reactions between $CO_2$, $H_2O$, and $CH_4$ within the geothermal aquifer. The mechanisms of $H_2$ generation remain to be

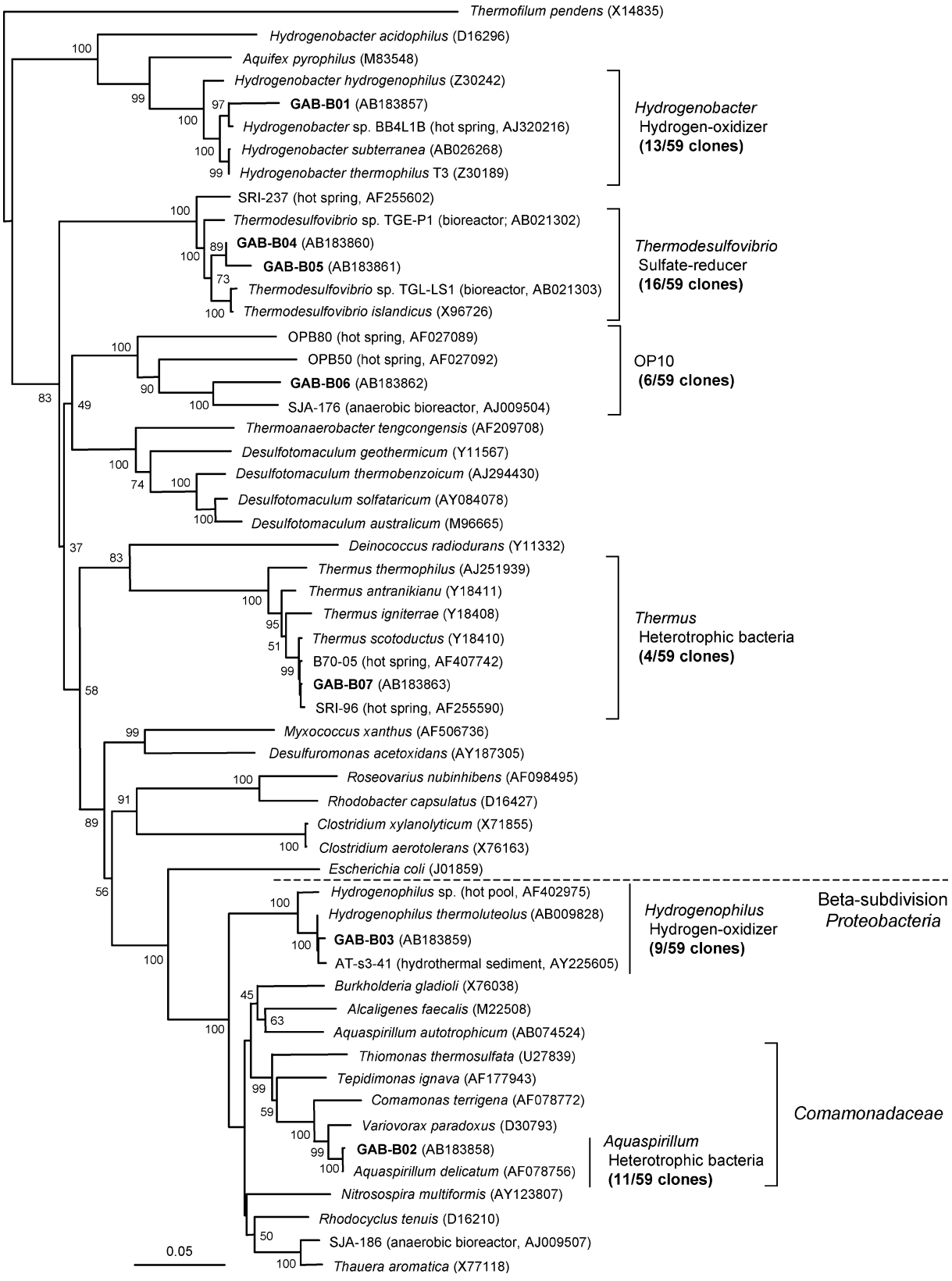


**Fig. 2** Molecular phylogenetic tree based on 16S rDNA sequences of seven representative clones in bacterial OTUs obtained from geothermal groundwater with the related species and environmental clones among the domain *Bacteria*. The tree was constructed by a neighbor-joining analysis with ca. 1,420 bp homologous positions of the rDNA sequences. The scale bar represents 0.05 nucleotide substitutions per sequence position

demonstrated; however, this study indicated the existence of an abundant microbial biomass that is supported by $H_2$ as a primary substrate within the geothermal aquifer.

The GAB was formed between 100 and 250 million years ago (Senior et al. 1978), and it has remained unchanged, since no volcanic activity is found in the area. The age of the oldest groundwater is estimated to be 2 million years (Elmore et al. 1979; Collon et al. 2000). In contrast to unstable environments such as hot springs and deep-sea hydrothermal vents, the deep GAB-aquifer has been extremely stable on the geological timescale. This aquifer is a suitable place for thermophilic microorganisms, and guarantees safe and permanent residence to these organisms. This isolated subsurface environment is practically independent of every circumstance observed at ground level. Inhabitants living in the deep GAB-aquifer would have the chance to survive certain global disasters which would cause serious damage to all life on the surface of the planet.

**Acknowledgments** This study was partly supported by the Japanese Ministry of Education, Culture, Sports, Science and Technology by a Grant-in aid for Creative Basic Research (14405016), ''Research for thermophilic and chemoautotrophic microorganisms inhibiting geothermal area in Oceania'' (a representative, Dr. Hiroyuki Yamamoto).

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